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Molecular Studies on Bromovirus Capsid Protein

I. Characterization of Cell-to-Cell Movement-Defective RNA3 Variants of Brome Mosaic Virus

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To evaluate the extent to which brome mosaic virus (BMV) coat protein (CP) gene is involved in the process of cell-to-cell movement, *Chenopodium quinoa* plants were coinoculated with BMV wild-type RNAs 1 and 2 and a RNA3 variant containing either the β-glucuronidase (GUS) gene in place of CP (B3/CP-GUS), to be subjected to GUS analysis, or a deletion in the CP gene to be analyzed by fluorescence *in situ* hybridization (FISH). Irrespective of time postinoculation, GUS expressed from the subgenomic mRNA of B3/CP-GUS was restricted to initially infected, single epidermal cells. The defective cell-to-cell movement exhibited by B3/CP-GUS was not complemented *in trans* when transcripts of B3ΔMP, a RNA3 variant capable of synthesizing functional wt CP but not movement protein (MP), were added to the inoculum. Application of FISH, a technique versatile in discriminating subliminal infections from efficient cell-to-cell spread in nonpermissive and permissive hosts, respectively, to leaves inoculated with BMV RNA3 variants defective in CP synthesis, confirmed that the resulting infections were subliminal. These data provide direct evidence for the requirement of an encapsidation-competent CP to be expressed in conjunction with a functional MP for efficient cell-to-cell spread of BMV.

INTRODUCTION

The ability of a virus to move from the initial site of infection to neighboring healthy cells in a susceptible host plant is an important prerequisite for establishing a successful infection. Many RNA plant viruses employ a viral-encoded movement protein (MP) to facilitate short distance or cell-to-cell spread (reviewed by Deom *et al.,* 1992; Lucas and Gilbertson, 1994). In the absence of a functional MP, the virus spread in a plant is restricted to the initially inoculated cells and the infection remains subliminal (Deom *et al.,* 1992). The most convincing evidence for the involvement of viral-encoded MP in cell-to-cell movement comes from studies conducted with tobacco mosaic tobamovirus (TMV), cucumber mosaic cucumovirus (CMV), and red clover necrotic mosaic dianthovirus. The MPs of these viruses have been shown to modify the size exclusion limits of the plasmodesmata and traffic themselves and the viral genomic RNA as a ribonucleoprotein complex across cell boundaries (Citovsky, 1993; Ding *et al.,* 1995a; Fujiwara *et al.,* 1993; Lucas *et al.,* 1995; Wolf *et al.,* 1989). In contrast to tobamo and dianthoviruses, the requirements for cell-to-cell movement for members of the como and nepoviruses are different. In these two groups, MP cooperates with the coat protein (CP) for viral cell-to-cell movement (Ritzenthaler *et al.,* 1995; Van Lent *et al.,* 1991). Electron microscopic studies performed on plants infected with como and nepoviruses have shown that infected cells contain unique tubular structures filled with virus-like particles that extend from the cell wall into the cytoplasm of neighboring cells (Ritzenthaler *et al.,* 1995; Van Lent *et al.,* 1991). Likewise, in tobacco etch potyvirus (Dolja *et al.,* 1995) and potato virus X (PVX; Baulcombe *et al.,* 1995; Chapman *et al.,* 1992; Oparka *et al.,* 1995) CP has been shown to be required for cell-to-cell movement. However, not all RNA viruses share the requirement for CP to achieve long distance movement, since the deletion of the CP gene from either tomato bushy stunt tombusvirus (Scholthof *et al.,* 1993) or barley stripe mosaic hordeivirus (Petty and Jackson, 1990) has no significant influence on long distance spread of these viruses in plants.

The process of cell-to-cell movement for plant viruses with a tripartite genome has been studied for members of the bromoviruses and the genetically related cucumoviruses. The genome organization of these two virus groups is very similar and viral genes are distributed among three RNA components. Viral replication is dependent on the nonstructural proteins encoded by RNAs 1 and 2 (Ahlquist, 1994; Nitta *et al.,* 1988; Palukaitis *et al.,* 1992). The two gene products encoded by the dicistronic RNA3 of bromo and cucumoviruses are dispensable for viral replication (Ahlquist, 1994; Palukaitis *et al.,* 1992). A nonstructural 3a protein encoded by the 5′ half of RNA3 shares a moderate amount of sequence similarity with MPs of several taxo-

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nomically distinct viruses (Allison *et al.,* 1989; Melcher, 1990). The structural CP gene encoded by the 3′ half of RNA3 is translated from the subgenomic RNA4 (Boccard and Baulcombe, 1993; Miller *et al.,* 1985). Although it has been shown that the 3a protein of CMV potentiates cell-to-cell trafficking of CMV RNA in transgenic tobacco plants (Ding *et al.,* 1995a), a functional analysis of CMV CP mutants indicated that, in addition to MP, CP is also involved in cell-to-cell movement (Boccard and Baulcombe, 1993; Suzuki *et al.,* 1991; Talianski and Garcia-Arenal, 1995). The bromovirus 3a gene (referred to as MP) is multifunctional. It is critical for cell-to-cell movement (Mise and Ahlquist, 1995; Mise *et al.,* 1993), dictates host specificity (De Jong and Ahlquist, 1991; De Jong *et al.,* 1995; Mise and Ahlquist, 1995), and modulates symptom expression in susceptible hosts (Fujita *et al.,* 1996; Rao and Grantham, 1995a). The role of bromovirus CP in this process is, however, not well understood. Flasinski *et al.* (1995) reported that brome mosaic virus (BMV) CP is not obligatory to elicit localized infections in *Chenopodium hybridum.* In contrast to these observations, several encapsidation-incompetent BMV CP mutants were unable to accumulate in inoculated leaves of barley (Rao and Grantham, 1995b; Sacher and Ahlquist, 1989) and also failed to elicit local lesions on several *Chenopodium* spp. (Rao and Grantham, 1995b), suggesting that CP is required to potentiate cell-to-cell movement along with the MP. To elucidate the role played by BMV CP in cell-to-cell movement, we employed two experimental approaches, an *in situ* histochemical β-glucuronidase (GUS) assay and fluorescence *in situ* hybridization (FISH), which allowed us to follow the spread of infection controlled by BMV CP variants.

MATERIALS AND METHODS

Plasmid constructions

Full-length cDNA clones corresponding to the three genomic RNAs of BMV, pT7B1, pT7B2, and pT7B3(-Tth), from which wild-type infectious RNAs 1 (B1), 2 (B2), and 3 (B3), respectively, can be transcribed *in vitro,* have been described previously (Dreher *et al.,* 1989; Rao *et al.,* 1989). Plasmid FCP2GUS (Mori *et al.,* 1993; renamed in this study pT7B3/CP-GUS; Fig. 1A), a derivative of BMV RNA3 cDNA, was provided by T. Okuno. A MP-defective clone of B3 was constructed by digesting pT7B3(-Tth) with *Cla*I (present at position 601; Fig. 1A) followed by treatment with *Bal*31 (Sambrook *et al.,* 1992). The resulting digestion products were ligated and transformed. A plasmid referred to as pT7B3ΔMP (Fig. 1A) was selected and used in subsequent studies. Plasmid pT7B3ΔMP/CP-GUS was constructed by digesting pT7B3/CP-GUS with *Bgl*II/*Kpn*I and the fragment containing the GUS gene was ligated to similarly treated pT7B3ΔMP (Fig. 1A). Plasmid pT7B3ΔBS (Fig. 1A) was constructed by sequentially digesting pT7B3 with *Bss*HII and *Stu*I (bases 1282–1781 deleted) and treating the vector product with mung bean nuclease prior to religation. Plasmid pT7B3SGI containing a ^{1242}G $\rightarrow$ ^{1242}U transversion at the subgenomic initiation site of RNA3 (Marsh *et al.,* 1991) was provided by T. C. Hall. Plasmids pT7B3/CP-GUS and all other B3 variants were linearized with *Eco*RI and *Bam*HI, respectively, prior to *in vitro* transcription.

In vitro transcriptions and biological assays

Full-length cDNA clones were transcribed *in vitro* in the presence of 7-methylguanosine(5′)triphospho(5′)guanosine cap with T7 RNA polymerase using a MEGAscript T7 kit (Ambion, Inc., Austin, TX). Isolation and transfection of barley (*Hordeum vulgare* cv. Dickson) protoplasts, the procedures used to extract total RNA, and analysis of RNA by Northern hybridization using riboprobes of desired specificity were performed as described previously (Rao *et al.,* 1994; Rao and Grantham, 1995a,b). For whole plant inoculations, *Nicotiana glutinosa* and *Chenopodium quinoa* plants were kept in the dark for at least 18 hr and mechanically inoculated as previously described (Rao *et al.,* 1994). Each experiment was repeated at least three to five times with independently synthesized *in vitro* transcripts. The inoculated plants were kept in the greenhouse at 25° for observation.

RT-PCR and sequence analysis of progeny RNA

Progeny viral RNA was suspended in sterile distilled water and subjected to reverse transcription-PCR as described previously (Rao *et al.,* 1994; Rao and Grantham, 1995a,b). The PCR products were either directly sequenced (Rao and Grantham, 1995b) or cloned into the *Sma*I site of pT7/T3*lac*Z and sequenced with Sequenase (Rao and Hall, 1993).

In vivo labeling and analysis of viral translation products

Proteins were labeled *in vivo* by the addition of 80 μCi of [^{35}S]methionine to culture medium which contained 1 $\times$ 10^5 transfected barley protoplasts previously irradiated with UV light (Sacher and Ahlquist, 1989). Cells were incubated for 24 hr and samples were suspended in SDS–PAGE sample buffer, denatured by boiling for 5 min, and fractionated on 12% polyacrylamide gels according to Laemmli (1970). Gels were then dried and exposed to Kodak BioMax film with an intensifying screen.

GUS assays

In situ GUS assays were done using a colorimetric substrate according to Lapidot *et al.* (1993). *C. quinoa* leaves were vacuum infiltrated with a solution containing 50 m*M* sodium phosphate, pH 7.0, 0.5 m*M* potassium ferricyanide, 0.5 m*M* potassium ferrocyanide, 1 m*M* EDTA, pH 8.0, 1.2 m*M* substrate 5-bromo-4-chloro-3-indo-

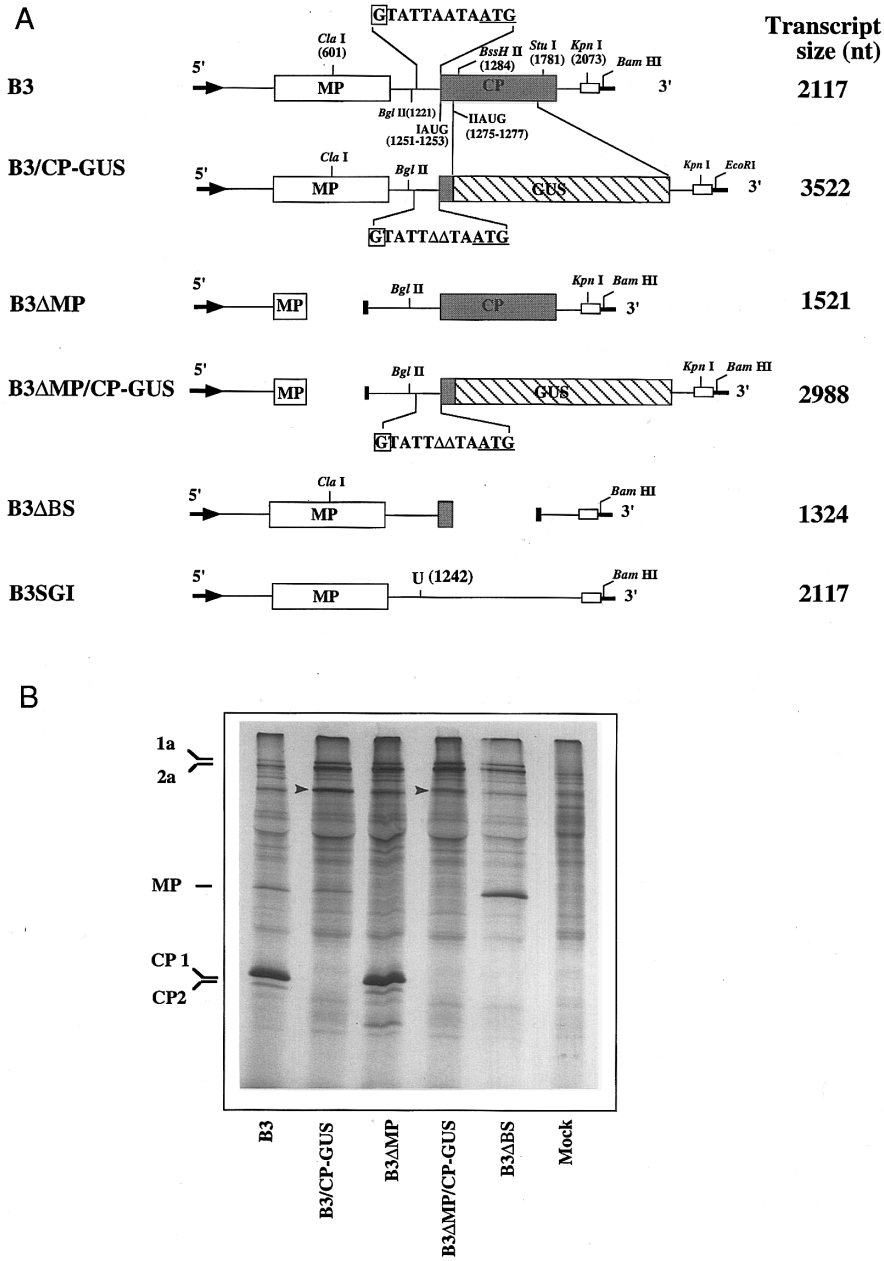


FIG. 1. Characteristics of BMV RNA3 (B3) variants. (A) Schematic representation of alterations introduced into the biologically active clone of wt B3. The genome organization of wt B3 is shown, with noncoding sequences represented as single lines and MP and CP genes as open and stippled boxes, respectively. The positions of selected restriction enzymes in B3 used to either introduce desired deletions or transfer genes between clones are shown. The locations of the normal AUG codon (IAUG) and the second AUG codon (IIAUG) of CP at positions 1251–1253 and 1275–1277, respectively, are indicated. The leader sequence 5′ to the normal AUG codon (underlined) with the first base of subgenomic RNA4 (boxed) is shown. In B3/CP-GUS, the GUS gene was fused to the second AUG codon. In this construct two adenosine residues present in the 5′ leader sequence to the first AUG codon were absent (shown as ΔΔ; Mise *et al.,* 1992). For B3ΔMP, the space indicates the extent of the deletion in the MP gene and the out-of-frame mutation created by *Bal*31 is indicated as a black box. B3ΔMP/CP-GUS was constructed by incorporating the GUS gene from B3/CP-GUS as a *Bgl*II and *Kpn*I fragment into B3ΔMP. In B3ΔBS, the space represents the extent of the deletion and the black box represents the position of the out-of-frame mutation. In B3SGI, the subgenomic initiator base ^{1242}G was mutated to ^{1242}U (Marsh *et al.,* 1991). (B) Autoradiograph of [^{35}S]methionine-labeled proteins extracted from transfected barley protoplasts and separated by SDS–PAGE. Positions of three nonstructural proteins (1a, 2a, and MP) and two coat proteins (CP1 and CP2) are marked on the left. Protoplasts were mock inoculated (far right lane) or coinoculated with wt B1 and B2 and the indicated B3 variants. Arrowheads indicate the position of GUS protein.

lyl-β-D-glucuronic acid, cyclohexylammonium salt (Gold Biotechnology, St. Louis, MO), and 0.5% Triton X-100. Chlorophyll was removed from leaves by soaking in a solution containing 42% ethanol, 10% formaldehyde, and 5% glacial acetic acid and then briefly in 70% ethanol (Lapidot *et al.,* 1993). Infection sites were identified by

the presence of an indigo precipitate. They were observed and photographed under bright-field optics using a Nikon photomicroscope.

Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization was adapted from Oh *et al.* (1995). One minute postinoculation, leaves were rinsed with sterile water to remove carborundum and any excess inoculum. Primary inoculated leaves of either *N. glutinosa* or *C. quinoa* were harvested at different time points and subjected to ethanol baths to remove chlorophyll. Leaves were treated with a pronase buffer (0.1 m*M* NaN_3, 0.1% SDS, 0.5 mg/ml Pronase; 10 m*M* EDTA, pH 8) followed by denaturation with 0.2 *N* HCl. After the leaves were rinsed with 2× SSC, they were mounted on acetate paper and baked at 80° in a vacuum oven. Prehybridization preceded hybridization with HPLC-purified fluorescein-labeled oligonucleotide probes (1.5 pmol/μl; Cruachem, Inc., Dulles, VA) complementary to a specific region in the CP gene (^{1577}dAGCAACACAAGCCTTAATCCTCCCA^{1553}), the MP gene (^{603}dGATTCCTACCGCTATCACAGCCGAT^{579}), or the 3′ homologous noncoding region (^{2106}dAGAGATTTACAGTGTTTTTCAACAC^{2082}). After hybridization, the following washes were performed: twice with 2× SSC at room temperature, twice with 2× SSC containing 0.1% SDS at 50°, and once with 2× SSC at room temperature. The leaf samples were examined under an epifluorescence microscope (Zeiss, Germany) with a standard fluorescence filter set (excitation filter BP 440-490 nm, barrier filter LP 520 nm) and photographed with Fujichrome 400 ASA film.

RESULTS

Characteristics of BMV RNA3 variants used in this study

In order to study the role of BMV CP in cell-to-cell movement, a series of B3 variants was constructed (Fig. 1A). All plasmids have the prefix T7 reflecting the presence of the T7 promoter. RNAs produced either by *in vitro* transcription or by virus replication are referred to by the plasmid name without the prefix. For example, plasmid pT7B3/CP-GUS, from which RNA designated B3/CP-GUS was transcribed (3522 nt; Fig. 1A), is characterized by the fusion of the entire GUS gene to the second AUG codon present at the eighth position in the CP ORF of B3 (Fig. 1A). In this plasmid, the leader sequence of the CP gene lacks two adenosine residues at position −3 and −4 relative to the first AUG codon, resulting in a change in the translational context and allowing elevated levels of GUS to be expressed from the second AUG codon (Mori *et al.,* 1993). Plasmid pT7B3ΔMP, from which RNA designated B3ΔMP was transcribed (1521 nt; Fig. 1A), is characterized by an out-of-frame deletion of 596 bases from the MP gene sequence. Plasmid pT7B3ΔMP/CP-GUS, from which RNA designated B3ΔMP/CP-GUS can be transcribed (2988 nt; Fig. 1A), was constructed by transferring the GUS gene from pT7B3/CP-GUS into pT7B3ΔMP. Plasmid pT7ΔBS, from which RNA designated B3ΔBS was transcribed, contained a frame-shift deletion of 499 bases between *Bss*hII and *Stu*I sites in the CP ORF (Fig. 1A). Plasmid pT7B3SGI contained a ^{1242}G → ^{1242}U transversion at the subgenomic initiation site of RNA3 (Fig. 1A) and therefore RNA4 cannot be synthesized (Marsh *et al.,* 1991).

All B3 variants replicated efficiently in the presence of wt B1 and B2 in barley protoplasts (data not shown) and synthesized proteins of expected sizes (Fig. 1B). The chimera B3/CP-GUS replicated efficiently in the presence of wt B1 and B2 despite it being 1405 nt longer than wt B3 (Mise *et al.,* 1993). Thus, cotransfection of wt B1 and B2 and either B3/CP-GUS or B3ΔMP/CP-GUS resulted in the efficient synthesis of GUS, which comigrated with protein present in mock-inoculated protoplasts (indicated by arrowheads in Fig. 1B). GUS synthesis in these samples was confirmed by the development of indigo precipitate within 2 hr of addition of GUS substrate (data not shown). As expected, protoplast transfections containing wt B1 + B2 + B3ΔMP resulted in expression of wt levels of CP but not MP (Fig. 1B). By contrast, wt B1 + B2 + B3ΔBS expressed wt levels of MP but not CP (Fig. 1B). Progeny (−) strands generated *in vivo* from transcripts of B3SGI, when cotransfected with wt B1 and B2, were defective as templates for the synthesis of the CP subgenomic messenger RNA4 and consequently no CP was synthesized (Marsh *et al.,* 1991; Rao and Grantham, 1995b).

Movement of B3/CP-GUS is restricted to single cells

Previous studies demonstrated that deletion or inactivation of the BMV CP gene completely abolished infection in barley and several *Chenopodium* spp., suggesting a role for CP in movement and symptom formation (Sacher and Ahlquist, 1989; Rao and Grantham, 1995b). To address whether BMV CP plays a critical role in cell-to-cell movement, young *C. quinoa* plants were mechanically inoculated with a mixture containing transcripts of wt B1 + B2 + B3/CP-GUS, and plants were observed for symptoms. Parallel inoculations of *C. quinoa* were also made with four control inocula containing wt B1 and B2 and the following B3 transcripts: (i) wt B3, (ii) B3ΔMP, (iii) B3ΔMP/CP-GUS, and (iv) B3ΔBS. *C. quinoa* plants inoculated with all three wt transcripts developed characteristic symptoms (Rao and Grantham, 1995b). These were typified by the development of local chlorotic lesions at 3–4 days postinoculation (dpi) followed by systemic mottling at 8 dpi (Table 1). None of the plants inoculated with wt B1 and B2 and B3/CP-GUS, B3ΔMP, B3ΔMP/CP-GUS, or B3ΔBS developed symptoms on ei-

TABLE 1

Analysis of GUS Activity in *C. quinoa* Inoculated with BMV RNA3 Variants

Inoculum[a]	Symptoms[b]		Hours postinoculation	GUS activity[c]		No. of infection sites examined[d]	No. of infected sites with indicated no. of cells[e]		
	I	S		I	S		1	2–3	4
B3/CP-GUS	−	−	6	+	−	8	8	0	0
			12	+	−	14	12	2	0
			24	+	−	31	29	1	1
			48	+	−	33	32	1	0
			72	+	−	44	43	1	0
			144	+	−	38	37	1	0
B3ΔMP/CP-GUS	−	−	6	+	−	6	6	0	0
			12	+	−	8	8	0	0
			24	+	−	12	10	1	1
			48	+	−	23	22	1	0
			72	+	−	31	29	1	1
			144	+	−	30	29	1	0
B3	+	+	NT	NT	NT	NT	NT	NT	NT
B3ΔMP	−	−	NT	NT	NT	NT	NT	NT	NT
B3ΔBS	−	−	NT	NT	NT	NT	NT	NT	NT

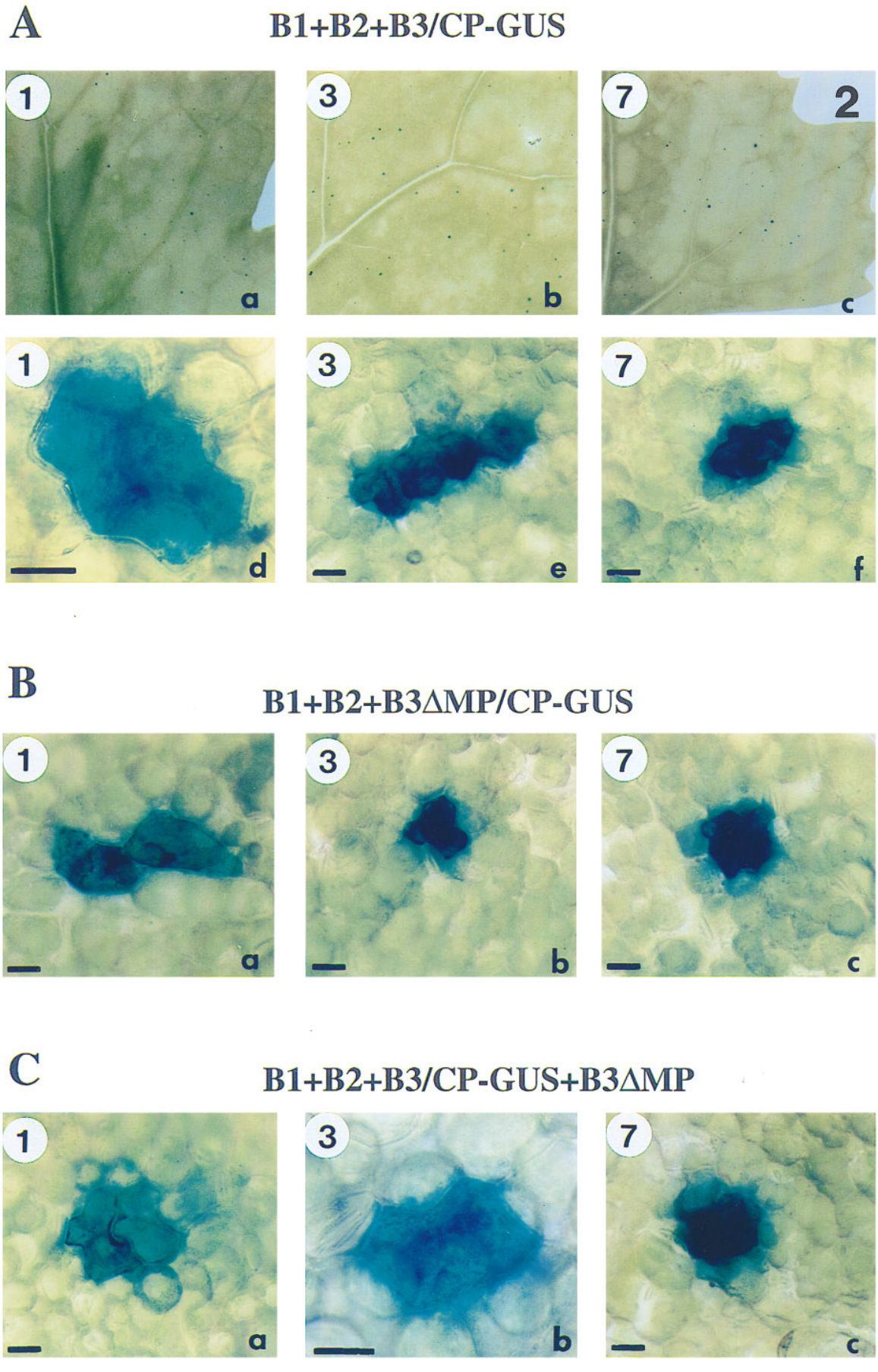
[a] Each inoculum (150 μg/ml) contained wt B1 and B2 in addition to the indicated B3 transcript.
[b] Presence (+) or absence (−) of symptoms characteristic of BMV on either inoculated (I) or systemic (S) leaves of *C. quinoa.*
[c] Presence (+) or absence (−) of GUS activity in either inoculated (I) or systemic (S) leaves; NT, not tested.
[d] Average number of infection foci from four independent experiments at each time point; NT, not tested.
[e] Infection sites were examined by microscopic visualization of GUS activity in primary inoculated leaves; NT, not tested.

ther inoculated or upper uninoculated leaves throughout the examination period (Table 1) and no progeny RNA were detected in these plants as tested by Northern blot analysis (data not shown).

To verify GUS activity in *C. quinoa* inoculated with wt B1 + B2 + B3/CP-GUS, primary leaves were excised at various intervals, vacuum infiltrated with GUS substrate, and periodically examined for indigo precipitation under the microscope. Since MP has been shown to be essential for cell-to-cell movement in bromoviruses (Mise and Ahlquist, 1995), primary leaves of *C. quinoa* inoculated with wt B1 + B2 + B3ΔMP/CP-GUS were also processed. It was anticipated that infections resulting from this chimera would not spread because of the absence of both MP and CP genes, thus serving as an effective negative control. In leaves inoculated with wt B1 + B2 + B3/CP-GUS an indigo color precipitate was detected as early as 6 hr pi when examined under the microscope (Table 1). Leaves processed at subsequent time points displayed infection foci as macroscopic pinpoint blue spots. The size and appearance of these spots were similar at each sampling time point (Fig. 2A, a–c). Microscopic examination revealed that the expression of GUS in the majority of infection site was restricted to single epidermal cells regardless of when samples were taken (Table 1; Fig. 2A, d and f). In a few sites, GUS expression was observed in two to four cells per infection site (Table 1; Fig. 2A, e). A similar profile of GUS activity was observed when primary leaves of *C. quinoa* were inoculated with the negative control wt B1 + B2 + B3ΔMP/CP-GUS (Table 1; Fig. 2B, a–c). These results suggest that CP is required for BMV to move beyond initially infected cells.

Defective cell-to-cell movement of B3/CP-GUS is not complemented *in trans*

To examine whether the defective cell-to-cell movement function exhibited by B3/CP-GUS can be restored by supplementing the CP *in trans,* capped transcripts of B3ΔMP (capable of synthesizing wt CP but not MP; Figs. 1A and 1B) were added to a mixture containing wt B1 + B2 + B3/CP-GUS and inoculated onto *C. quinoa* plants. As a control, plants were also inoculated with a mixture of wt B1 + B2 + B3ΔMP + B3ΔBS. Throughout the examination period, plants inoculated with wt B1 + B2 + B3/CP-GUS + B3ΔMP displayed neither local nor systemic symptoms (Table 2). However, when inoculated leaves were infiltrated with GUS substrate, several pinpoint blue spots were observed. The sizes of these blue spots were indistinguishable from those observed on plants inoculated with either wt B1 + B2 + B3/CP-GUS (Fig. 2A, a–c) or B3ΔMP/CP-GUS, suggesting that infection did not spread to adjacent cells with time. GUS activity in these infection foci was restricted to one or two epidermal cells (Fig. 2C, a–c). No GUS activity was observed in systemic leaves of these plants (Table 2). Plants inoculated with wt B1 + B2 + B3ΔMP or B3ΔBS individually failed to acquire any symptoms, and no progeny RNA was detected in Northern blots (Table 2). Simi-


A
B1+B2+B3/CP-GUS
1
a
3
b
7
2
c
1
d
3
e
7
f
B
B1+B2+B3ΔMP/CP-GUS
1
a
3
b
7
c
C
B1+B2+B3/CP-GUS+B3ΔMP
1
a
3
b
7
c

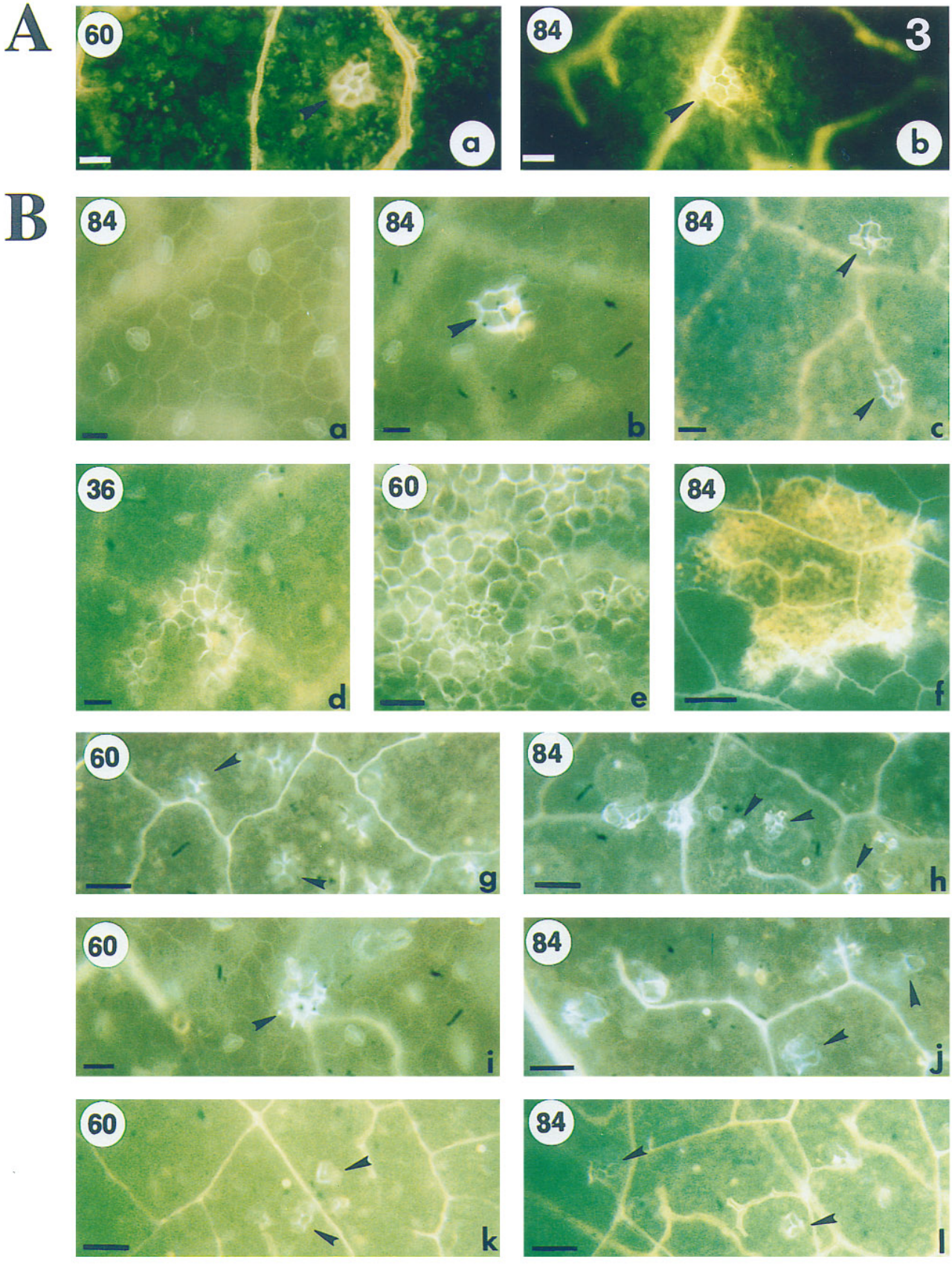

A
60
a
84
3
b
B
84
a
84
b
84
c
36
d
60
e
84
f
60
g
84
h
60
i
84
j
60
k
84
l

TABLE 2

Analysis of Complementation between BMV RNA3 Variants

Inoculum[a]	Symptoms[b]		GUS activity[c]		Northern blots[d]		Progeny sequence[e]
	I	S	I	S	I	S	
B3/CP-GUS + B3ΔMP	−	−	+	−	−	−	None
B3ΔBS	−	−	NT	NT	−	−	None
B3ΔMP	−	−	NT	NT	−	−	None
B3ΔMP + B3ΔBS	+[f]	+[f]	NT	NT	+	+	Wild type

[a] Each inoculum (150 μg/ml) contained wt B1 and B2 in addition to the indicated B3 variant(s).

[b] Presence (+) or absence (−) of symptoms characteristic of BMV on either inoculated (I) or systemic (S) leaves of *C. quinoa.*

[c] Presence (+) or absence (−) of GUS activity in either inoculated (I) or systemic (S) leaves; NT, not tested.

[d] BMV RNAs were detected by Northern hybridization. I, inoculated leaves; S, systemic leaves; +, presence of BMV RNAs; −, absence of BMV RNAs.

[e] Sequence of the progeny RNA was determined as described under Materials and Methods.

[f] Symptoms appeared on inoculated and noninoculated systemic leaves only when recombination had occurred (see text for details).

larly, the control plants inoculated with wt B1 + B2 + B3ΔMP + B3ΔBS did not develop local lesions by 3–4 dpi. However, by 7 dpi, chlorotic local lesions characteristic of wt BMV infection developed in two of eight of these plants. These lesions rapidly coalesced and, subsequently, plants developed systemic mottling symptoms (Table 2). Northern blot analysis of total RNA isolated from these symptomatic primary and systemic leaves revealed an RNA profile similar to that of a wt infection (Table 2). Sequence analysis of progeny RNA3 revealed that infection was restored due to the regeneration of a functional wt RNA3 by recombination between the two RNA3 variants supplied in the inoculum (Table 2; Allison *et al.,* 1990).

Characterization of subliminal infections using FISH technique

The above data demonstrated that synthesis of GUS provides a highly sensitive marker for infected individual cells. One possible explanation for the confinement of infection of wt B1 + B2 + B3/CP-GUS to single cells could be due to the increased size of the chimeric RNA (Fig. 1A). To show that the defective cell-to-cell movement exhibited by B3/CP-GUS is primarily due to the lack of CP and not due to the size of the GUS chimera, we utilized FISH. Prior to analyzing the cell-to-cell movement of CP-defective variants by FISH, the ability of this technique to distinguish subliminal infections from efficient cell-to-cell spread was tested by inoculating permissive and nonpermissive hosts with a series of inocula. *N. glutinosa* plants were inoculated with a mixture containing all three BMV wt transcripts. Since *N. glutinosa* is a nonpermissive host for BMV (Lane, 1981), it was anticipated that infections resulting from the wt inoculum would remain subliminal. To characterize subliminal infections in a permissive host, *C. quinoa* plants were inoculated with two different inocula, one containing wt B1 + B2 and the other containing only wt B3. It was expected that in plants inoculated with wt B1 + B2 alone, replica-

FIG. 2. Visualization of GUS activity in the primary leaves of *C. quinoa* inoculated with B3 variants. Plants were inoculated with a mixture of RNA transcripts (shown above) at a concentration of 150 μg/ml. Whole leaves were vacuum infiltrated with the histochemical GUS substrate X-glucuronidase at days postinoculation indicated at the top left. In A, the photographs represent macroscopic images of infiltrated *C. quinoa* leaves (a–c). There was no spread of GUS into adjacent cells over time. Images shown in d through f represent the microscopic examination of infected primary leaves of *C. quinoa.* Images shown in d and f are single epidermal cells infected with wt B1 + B2 + B3/CP-GUS at 1 and 7 dpi, respectively. A cluster of three epidermal cells representing a multiple cell infection at 3 dpi is shown in e. In B, images depict two infected cells (a) and single epidermal cells (b and c) following inoculation with wt B1 + B2 + B3ΔMP/CP-GUS. In C, images demonstrate the lack of complementation between wt B1 + B2 + B3/CP-GUS and B3ΔMP. Representative examples of simultaneous infection of two cells (a) and a single epidermal cell (b and c) are shown. Bar, 50 μm.

FIG. 3. Epifluorescent microscopic analysis of cell-to-cell movement in *C. quinoa* leaves subjected to FISH. In A, *N. glutinosa* leaves inoculated with B1 + B2 + B3 have subliminal infection sites (indicated by arrowheads) at 60 (a) and 84 (b) hr pi. In B, a mock-inoculated leaf of *C. quinoa* at 84 hr pi showing autofluorescence of veins and stomata is shown in (a). Subliminal infections resulting from inoculation of wt B1 + B2 (b) and wt B3 alone (c) at 84 hr pi are shown (arrowheads point to two individual sites of three or four fluorescing cells). Efficient cell-to-cell movement of wt BMV in leaves at 36 (d) and 60 hr pi (e) is shown. In (f), a single chlorotic local lesion formed at 84 hr pi caused by wt infection, encompassing several hundred fluorescent cells, is shown. Subliminal infection foci resulting from inoculation with wt B1 + B2 + B3ΔBS (g and h), wt B1 + B2 + B3-SGI (i and j), and wt B1 + B2 + B3ΔMP (k and l) at 60 and 84 hr pi are indicated by arrowheads in each image. In each case, distinct infection foci containing <5 cells are evident. Bar, 500 μm except in B, f, bar, 250 μm. Fluorescein-tagged oligonucleotide probes used to hybridize leaf samples shown in A and B are described in the text. Note that irrespective of the leaf sample, the fluorescing signal from cells containing viral RNA is significantly brighter than the autofluorescence exhibited by veins and stomata.

TABLE 3

Analysis of Cell-to-Cell Movement of BMV RNA in *N. glutinosa* and *C. quinoa* by Fluorescence *in situ* Hybridization

Inoculum[a]	Symptoms[b] I	Symptoms[b] S	Fluor. probe[c]	Hours postinoculation	No. of infection sites examined[d]	No. of infected sites with indicated no. of cells[e] 1–3	4–6	7–9	10–20	>20
N. glutinosa										
B1 + B2 + B3	−	−	MP	60	3	3	0	0	0	0
				84	5	5	0	0	0	0
C. quinoa										
B1 + B2	−	−	3′ end	36	0	0	0	0	0	0
				60	7	6	1	0	0	0
				84	22	16	6	0	0	0
B3	−	−	MP	36	3	3	0	0	0	0
				60	5	5	0	0	0	0
				84	22	17	5	0	0	0
B1 + B2 + B3	+	+	MP	36	4	0	2	1	1	0
				60	83	0	11	27	26	19
				84	16	0	2	3	7	4
B1 + B2 + B3/ΔMP	−	−	CP	36	0	0	0	0	0	0
				60	47	30	11	6	0	0
				84	90	70	17	3	0	0
B1 + B2 + B3/ΔBS	−	−	MP	36	2	1	1	0	0	0
				60	27	18	9	0	0	0
				84	20	15	5	0	0	0
B1 + B2 + B3SGI	−	−	MP	36	0	0	0	0	0	0
				60	10	8	2	0	0	0
				84	41	29	10	2	0	0

Note. Number of infection sites indicates averages taken from four independent experiments.

[a] All inocula were of 150 μg/ml concentration.

[b] Presence (+) or absence (−) of symptoms characteristic of BMV (Rao and Grantham, 1995b) on either inoculated (I) or systemic (S) leaves.

[c] Fluorescein-tagged oligonucleotide probes of indicated specificity were used to detect the corresponding inoculum.

[d] Infection sites were examined and counted under an epifluorescence microscope.

[e] Number of fluorescent cells per infection site on the inoculated leaves of *C. quinoa* and *N. glutinosa*.

tion would occur in initially infected cells but that infection would not spread in the absence of wt B3. Similarly, in plants inoculated with wt B3 transcript alone, B3 would neither replicate nor spread from the initial sites of introduction and thus any detected B3 in these plants would represent input inoculum only. To observe efficient cell-to-cell spread and to serve as a positive control, *C. quinoa* plants were inoculated with all three wt transcripts. Plants inoculated with only buffer served as mock controls.

Neither *N. glutinosa* plants inoculated with all three wt BMV transcripts nor *C. quinoa* inoculated with either wt B1 + B2 or wt B3 alone displayed any symptoms in either inoculated or uninoculated upper leaves (Table 3). By contrast, *C. quinoa* plants inoculated with all three BMV wt transcripts developed characteristic local lesions 4–5 dpi and developed systemic mottling symptoms 8 dpi (Table 3). To examine the spread of infection in each case, leaves of *N. glutinosa* or *C. quinoa* were harvested at various time points and prepared for hybridization with fluorescein-tagged oligonucleotide probes complementary to a desired region of the BMV genome. Since all four BMV RNAs contain a highly conserved 3′ tRNA-like sequence, a single probe complementary to this region could have been used. However, the application of such a probe would prevent the discrimination of subliminal infections, caused by B3 variants, from cells receiving either individual or a combination of any two RNA components. Therefore, three individual probes were used in this study. These were: (i) a probe complementary to a sequence within the MP ORF (designated MP probe; Table 3), which was used to detect B3 in leaves inoculated with all three wt transcripts (B1 + B2 + B3), wt B3 alone, and wt B1 + B2 + B3 variants expressing MP but not CP (i.e., wt B1 + B2 + B3/ΔBS and wt B1 + B2 + B3SGI); (ii) a probe complementary to the conserved 3′ end (designated 3′ end probe; Table 3), which was used to detect RNAs in leaves inoculated with wt B1 + B2; and (iii) a probe complementary to a region within the CP ORF (designated CP probe; Table 3), which was used to detect RNAs in leaves inoculated with wt B1 + B2 + B3/ΔMP. Results are summarized in Table 3 and representative examples are shown in Fig. 3.

When viewed under an epifluorescence microscope, mock-inoculated leaves had auto-fluorescing veins and stomata (Fig. 3B, a). Leaves treated with a fluorescein-tagged viral RNA-specific probe exhibited the following characteristics: (i) damaged areas, containing ruptured

cells with ragged edges, having a nebular appearance (Cooper *et al.,* 1996) and (ii) infected areas, where a specific probe had hybridized to viral target RNA, containing intact cells that were globular and had defined boundaries (Cooper *et al.,* 1996; for example Fig. 3B, b and e). When leaves of *N. glutinosa* inoculated with all three wt BMV transcripts were examined under an epifluorescence microscope, infection sites contained one to three cells at all sampling times (Fig. 3A, a and b; Table 3). Similar analysis of *C. quinoa* leaves inoculated with either wt B1 + B2 or wt B3 alone exhibited a majority of sites with one to three cells (Fig. 3B, b and c; Table 3). Since wt B3 cannot replicate in the absence of wt B1 and B2, the fluorescing sites in leaves inoculated with wt B3 alone represent input inoculum (Fig. 3B, c) and indicate that B3 is stable in plants for at least 84 hr, possibly due to its secondary structure. This observation is in agreement with previous reports in which a B2 variant, defective in replication due to the absence of the entire 3′ tRNA-like end, when coinoculated with wt B1 and B3 (Rao and Hall, 1990) or individual BMV RNA components inoculated alone, persisted for long periods of time in protoplasts (Rao *et al.,* 1990). Conversely, *C. quinoa* leaves inoculated with all three wt transcripts exhibited infection sites which expanded with time to encompass many cells (Fig. 3B, d–f; Table 3). At 84 hr pi, chlorotic local lesions which had developed in wt-inoculated *C. quinoa* leaves represented infection foci that involved several hundred fluorescing cells (Fig. 3B, f; Table 3), indicative of efficient cell-to-cell movement. Large areas of cells exhibiting fluorescence were also observed in uninoculated systemic leaves 7 dpi (data not shown). These observations clearly demonstrate that the FISH technique is suitable for discriminating subliminal infections from efficient cell-to-cell spread.

Restricted movement of BMV CP-defective variants

To examine the ability of CP defective variants of BMV to mediate cell-to-cell spread, primary leaves of *C. quinoa* were inoculated with wt B1 + B2 + B3ΔBS or B3SGI and subjected to FISH analysis. Since the deletion of MP inhibits cell-to-cell spread in bromoviruses (Mise *et al.,* 1993), plants were inoculated with wt B1 + B2 + B3ΔMP (Fig. 1A), a MP-defective variant of B3, as a control. Plants inoculated individually with each of the three inocula, wt B1 + B2 and B3ΔBS, B3SGI, or B3ΔMP, remained symptomless (Table 3). Leaves inoculated with wt B1 + B2 + B3ΔBS or B3SGI were hybridized with a fluorescein-tagged oligonucleotide probe complementary to a sequence within the MP ORF, whereas leaves inoculated with wt B1 + B2 + B3ΔMP were hybridized with an oligonucleotide probe complementary to a sequence within the CP ORF. Irrespective of time after inoculation, the infections resulting from wt B1 + B2 + B3ΔBS (Fig. 3B, g and h), B3SGI (Fig. 3B, i and j), or B3ΔMP (Fig. 3B, k and l) were of a subliminal nature and were indistinguishable from subliminal infections caused in the nonpermissive host by wt inocula (Fig. 3A, a and b) or in the permissive host by inocula containing wt B1 + B2 (Fig. 3B, b) or wt B3 alone (Fig. 3B, c) (Table 3). These observations clearly demonstrate that mutants incapable of synthesizing encapsidation-competent CP are defective in cell-to-cell movement and subsequent long distance movement, despite having intact MP.

DISCUSSION

In order to examine the role of BMV CP in viral spread in plants, we analyzed the movement behavior of several BMV RNA3 variants defective in the synthesis of an encapsidation-incompetent CP using two sensitive *in situ* assays, GUS and FISH (Figs. 2 and 3; Tables 1 and 3). Previously, Mise and Ahlquist (1995) demonstrated the requirement of the 3a gene of bromoviruses for cell-to-cell movement; however, the role played by CP in movement was not examined, since this part of the gene remained unaltered in their studies. The absence of cell-to-cell movement manifested by BMV CP variants examined in this study suggests that the presence of MP alone is not sufficient to support cell-to-cell spread of BMV infection. This is evidenced by the evaluation of the behavior of B3/CP-GUS (Fig. 2; Table 1) and by the application of FISH to other CP-defective mutants (B3ΔBS, B3SGI), which failed to move from cell to cell despite having an intact MP (Fig. 3B, g–j; Table 3). The lack of cell-to-cell movement exhibited by wt B1 + B2 + B3/CP-GUS (i) is not due to defective replication since this chimera was found to replicate efficiently in protoplasts (Mori *et al.,* 1993) and (ii) cannot be attributed to the increased size of the GUS chimera (Fig. 1A), since infections resulting from the inoculation of a BMV RNA3 chimera in which the green fluorescent protein gene, which is equivalent in size to that of BMV CP gene, was substituted for the CP, were also restricted to one or two cells (Rao, unpublished data). Although the majority of infection sites induced by wt B1 + B2 + B3/CP-GUS chimera consisted of single, epidermal cell infections, a low number of infection sites consisting of two to four cells were also observed (Fig. 2A, e; Table 1). Such infection sites were also detected in leaf samples inoculated with wt B1 + B2 + B3ΔMP/CP-GUS (Fig. 2B, a), a construct that was expected not to support cell-to-cell movement. Therefore, development of these subliminal infections of two to four cells could have either resulted from mechanical inoculation or occurred due to nonspecific passive movement. Similar multiple cell infections were observed following the inoculation of MP-defective variants of CCMV (Mise and Ahlquist, 1995) and TMV (Nishiguchi *et al.,* 1980; Cooper *et al.,* 1996) and of CP-defective variants of PVX (Baulcombe *et al.,* 1995; Oparka *et al.,* 1995).

The manifestation of a visible local lesion represents cell-to-cell spread. Each lesion comprises an infection center, caused by introduction of viral RNA, and an area of surrounding cells into which the invading virus has actively moved (reviewed by Goodman and Novacky, 1994). In previous studies (Rao and Grantham, 1995b), BMV RNA3 variants that failed to induce local lesions in *Chenopodium* spp. were also those which failed to produce a functional, encapsidation-competent CP. Recent additional infectivity assays in *C. quinoa* have shown that several CP amino-terminal deletion mutants with weakened RNA–CP interactions induced local lesions, but did not move systemically, whereas mutants that are incapable of forming virions were noninfectious (Rao and Grantham, 1996). Based on these findings, we surmise that infectivity is dependent on efficient interaction between the CP and the RNA and subsequent virion formation. This conjecture was further supported by a recent study by Kasteel *et al.* (1996). They observed the formation of tubular structures filled with BMV virions in transfected protoplasts, similar to those induced by comoviruses (Van Lent *et al.,* 1991) and nepoviruses (Ritzenthaler *et al.,* 1995), implying BMV is transported between cells as intact virions.

Previous studies demonstrated that encapsidation-competent CP is required for local spread in barley plants (Rao and Grantham, 1995b; Sacher and Ahlquist, 1989) or to elicit local lesions in several *Chenopodium* spp. (Rao and Grantham, 1995b). However, Flasinski *et al.* (1995) reported that BMV CP is redundant for local lesion production in *C. hybridum.* We wish to provide the following explanations for the discrepancy. BMV induces distinguishable phenotypes in the purple and green varieties of *C. hybridum.* In the purple variety, the virus elicits necrotic local lesions, signifying a hypersensitive response, without invading the host systemically (Rochow, 1959; Verduin, 1978; Rao *et al.,* 1990); whereas in the green variety BMV induces chlorotic local lesions followed by systemic mottling (Verduin, 1978; Rao and Grantham, 1995b). In contrast to these findings, Flasinski *et al.* (1995) reported that in the green variety of *C. hybridum* BMV elicits necrotic local lesions (as opposed to previously reported chlorotic lesions) followed by systemic infections. So it is likely that the necrosis seen by Flasinski *et al.* (1995) in the green variety could have been the result of either inoculation damage or some other aberration that was not a true symptom for the green variety. Furthermore, their observations were derived from inoculations containing high concentrations of RNA transcripts. Their standard inoculum contained a mixture of three BMV RNA transcripts (in 20 μl) derived from 1 μg each of linearized template DNA. Since 1 μg of template could potentially yield at least 5–10 μg RNA transcripts, their inoculum dose thus contained 0.75–1.5 mg/ml, which is at least 5–10 times more than the amount we routinely use in our inoculations. Truly, if the BMV CP was not essential for local lesion induction or cell-to-cell movement as Flasinski *et al.* (1995) suggested, they should have seen the true-to-type symptoms of BMV infection on green variety with their standard inoculum dose. But this was not the case. In their experiments local lesions developed only when 5 times the standard amount of RNA transcripts of CP-defective mutants was added to their inoculation mixture, although the rationale for increasing the concentration of CP-defective variants was not obvious. Therefore, it is likely that the BMV RNA recovered from inoculated leaves of *C. hybridum* in their experiments represents the RNA replicating in multiple initially infected cells from the heavily dosed inoculum rather than from local lesions specifically induced by BMV infection.

The lack of complementation between B3/CP-GUS and B3ΔMP further exemplifies the critical role played by BMV CP during the infection process. We envisioned that wt CP, synthesized from the subgenomic RNA of B3ΔMP, would *trans*-encapsidate progeny RNA and potentiate the movement of B3/CP-GUS. We offer two explanations for the lack of expected complementation. First, it is likely that one variant RNA3 is able to out compete the other. A second possibility is the absence of virions containing viral RNA which is essential for cell-to-cell movement (Kasteel *et al.,* 1996). Although the CP binding sites on BMV RNA3 have not been identified, it is possible that such sites are localized within the sequence encoding the CP gene. Therefore, wt CP translated from the subgenomic mRNA of B3ΔMP cannot interact with B3/CP-GUS to assemble into virions, since it lacks the CP coding sequence. This would also explain why B3ΔBS and B3ΔMP variants or similar variant clones of CCMV RNA3 (Allison *et al.,* 1990) failed to complement each other *in trans* when coinoculated to susceptible plants. In our experiments, infections occurred only when B3ΔBS and B3ΔMP recombined to generate functional MP and CP ORFs on the same RNA3 molecule. The restoration of infectivity is not due to contamination, since independent inoculations containing either B3ΔBS or B3ΔMP were noninfectious (Table 2). Similar observations were reported for CCMV (Allison *et al.,* 1990). Conversely, in several repeated experiments, no recombination occurred in plants coinoculated with B3/CP-GUS and B3ΔMP (Table 3). Although the reasons for the lack of recombination in these experiments is unknown, we speculate that replacement of CP with GUS must have altered the secondary structure of the chimeric RNA such that the polymerase was unable to switch templates during copying, a mechanism favored for RNA recombination (Kirkegaard and Baltimore, 1986).

While several studies have implied that MP and CP may be involved in cell-to-cell and long distance movement, the participation of other viral-encoded genes in this process has been noted for BMV and CMV (Ding *et al.,* 1995b; Gal-On *et al.,* 1994; Traynor *et al.,* 1991). Se-

lected BMV replicase mutants, capable of efficient replication in protoplasts, failed to support systemic infection in barley (Traynor *et al.,* 1991). Since these mutants were not defective in packaging BMV genomic RNAs, the impaired systemic movement was directly regulated by viral replicase genes. Similarly, in CMV, long distance movement is also regulated by CMV RNA1 (Gal-On *et al.,* 1994) as well as by the RNA2b gene (Ding *et al.,* 1995b). These observations, together with those presented in this study, suggest that in BMV all viral genes are involved in the overall spread of infection. Thus the effective movement of BMV, as well as other plant viruses, is dependent on a cooperative interaction between virus-encoded proteins and host proteins (Heinlein *et al.,* 1995; McLean *et al.,* 1995).

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